ANALYSIS OF THE DOSAGE COMPENSATION OF A SPECIFIC TRANSCRIPT IN *DROSOPHILA MELANOGASTER*

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ABSTRACT

The amount of steady-state level RNA complementary to the *X*-linked salivary gland secretion polypeptide gene *Sgs-4* was measured in male and female third-instar larvae carrying one or two doses of a wild-type allele of the gene. RNA levels were found to be compensated in normal one-dose males and two-dose females and to be dosage-dependent within each sex. The presence of mutant alleles of *male-less* (*mle*) was found to reduce the level of *Sgs-4* transcripts in males. These results support the contentions that dosage compensation is mediated by regulating the level of *X*-linked gene transcripts and that a product of the mle^+ gene is involved in this process.

DOSAGE compensation in Drosophila describes the phenomenon whereby males with a single *X*-chromosome are able to synthesize amounts of *X*-linked gene products that are relatively equivalent to those produced by females with two *X*-chromosomes, both of which are transcriptionally active (see LUCCHESI 1977; STEWART and MERRIAM 1980; LUCCHESI 1983; and BAKER and BELOTE 1983 for reviews).

Paralleling advances in technology, dosage compensation has been studied by four methods. Originally it was described by MULLER (1932) as an equalization of gross phenotypic expression of *X*-linked genes. Later, the dosage compensation of *X*-linked proteins was examined either by measuring their enzymatic activities (SEECOF, KAPLAN and FUTCH 1969; LUCCHESI and RAWLS 1973; FAIZULLIN and GVOZDEV 1973; LUCCHESI, RAWLS and MARONI 1974; BELOTE and LUCCHESI 1980a) or by measuring their quantities directly in gels (KORGE 1975, 1981; WILLIAMSON and BENTLEY 1983). MUKHERJEE and BEERMAN (1965) introduced the technique of transcription autoradiography to the study of the phenomenon, and it was subsequently utilized by others to quantitate chromosomal RNA synthesis under varying genetic conditions (MARONI and PLAUT 1973; ANANIEV, FAIZULLIN and GVOZDEV 1974; LUCCHESI, BELOTE and MARONI 1977; MARONI and LUCCHESI 1980; BELOTE and LUCCHESI 1980a; LUCCHESI and SKRIPSKY 1981). Finally, the levels of specific *X*-linked RNAs

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have been studied either by labeling the RNAs as they are transcribed and hybridizing the specific RNA to a filter-bound cloned DNA (BIRCHLER, OWENBY and JACOBSON 1982) or by blotting RNA to nitrocellulose filters and hybridizing with a specific labeled DNA (S. K. BECKENDORF, unpublished results; GANGULY, GANGULY and MANNING, 1985).

Recently, a small group of autosomal genes, characterized by the male-specific lethality of their mutant alleles, has been correlated to the mechanism of dosage compensation (BELOTE and LUCCHESI 1980a,b). Homozygous mutant males exhibit significantly reduced levels of *X*-linked enzymes in relation to the levels of autosomal enzymes. Furthermore, autoradiographic monitoring of total chromosomal RNA synthesis on larval polytene chromosomes of males homozygous for one of these mutations reveals a reduced rate of synthesis along the *X* chromosome.

In this paper we establish the effect of mutant alleles of one of these genes, *male-less* (*mle*, *2*-55.8), on the level of a specific *X*-linked transcript. The transcript in question is that coded by the salivary gland secretion polypeptide gene *Sgs-4* (KORGE 1975; BECKENDORF and KAFATOS 1976; MUSKAVITCH and HOGNESS 1980; MCGINNIS, FARRELL and BECKENDORF 1980). As an internal control, the RNA encoded by the autosomal *Sgs-3* gene (KORGE 1975; BECKENDORF and KAFATOS 1976; MEYEROWITZ and HOGNESS 1982) was quantitated. These RNAs are coordinately expressed from the middle of the third larval instar to the prepupal stage. Our results show a moderate yet significant reduction in the relative level of the *X*-linked transcript in mutant males.

MATERIALS AND METHODS

Third instar larvae to be used for salivary gland dissection were selected on the basis of sex, developmental stage and phenotype. Males and females were distinguished on the basis of gonad size and were staged by the extent of their fat body development. In previous experiments using a known staging technique (MARONI and STAMEY 1983), it was observed that as larvae grow older their fat body becomes more extensive and more opaque until it obscures all but a small posteroventral portion of the gut. The larval marker mutation *Bc* was used to differentiate homozygous mutant and heterozygous individuals. [*Bc* is a dominant larval mutation on the second chromosome that produces black cells as described by E. H. GRELL (1969).] The DNA used as a hybridization probe for steady-state *Sgs-3* RNA and *Sgs-3* DNA, λcDM2008 (MEYEROWITZ and HOGNESS 1982), was a gift of STEPHEN BECKENDORF. The DNA used as a probe for steady-state *Sgs-4* RNA, pkdm4B1 (MUSKAVITCH and HOGNESS 1980), was a gift of MARC MUSKAVITCH. The cloned DNA used as a probe for *Sgs-4* DNA was pOR H3.4, which is a *Hin*dIII genomic fragment inserted into pBR322. This clone was also a gift of STEPHEN BECKENDORF.

Total nucleic acid was extracted by the method of MEYEROWITZ and HOGNESS (1982). RNA was isolated from total nucleic acid by the procedure of TULLIS and RUBIN (1980), and DNA was isolated by digestion with RNase A (2 mg/ml of Tris-EDTA buffer for 2 h at 37°). RNA gels were prepared according to GOLDBERG (1980) and were blotted according to SOUTHERN (1975). RNA dot blots were prepared according to THOMAS (1983). RNAs were treated so that 5 μl would deliver 4 μg of RNA to the filter, and this solution could be diluted subsequently using 0.1% SDS. DNA dot blots were prepared according to the procedure of KAFATOS, JONES and EFSTRADIADIS (1979). Again, DNAs were treated so that 5 μl would contain 4 μg, and subsequent dilutions were made using 1 M NH_4OAc.

Replica dot blot filters were prepared using total RNA isolated from salivary glands of staged third-instar larvae of the various genotypes. For each sex of each sibling pair of genotypes, a number of salivary gland samples were extracted, and the RNA from each sample was quantitated and then blotted to nitrocellulose filters in an array of dots that contained 4, 2, 1 and 0.5 μg of RNA. For each sibling pair of genotypes, all of the separately prepared RNA samples were blotted to the same replica pair of filters. One filter was hybridized to ^{32}P-labeled pkdm4B1 DNA, and its replica was hybridized to ^{32}P-labeled λcDm2008 DNA. Each of these two probes hybridizes to its homologous RNA without any cross-hybridization to the other RNA (MUSKAVITCH and HOGNESS 1980; MEYEROWITZ and HOGNESS 1982). The resulting radioactive dots were cut out of the filters and counted. The ratio of counts hybridized to *Sgs-4* RNA to counts hybridized to *Sgs-3* RNA was calculated for each replica pair of dots. This ratio was used in the comparison of the relative levels of expression of *Sgs-4* between males and females.

The counts which hybridized to dots made with 4 μg of total RNA were compared to the counts which hybridized to dots made with 2 μg of total RNA in order to demonstrate quantitative hybridization. This quantitative comparison of counts was made separately for the *Sgs-4* and *Sgs-3* probed filters; and in order for the results to be used, the ratio derived from the *Sgs-4* probed filter had to be within 5% of the ratio derived from the *Sgs-3* probed filter. This value was arbitrarily chosen as being indicative of relatively similar levels of quantitative hybridization on the replica filters. The experimental results were calculated using only one quantitative class from each replica pair of filters, *i.e.*, either the results derived from the 4 μg hybrids or the 2 μg hybrids were used, but not both, in order to avoid committing pseudoreplication.

RNA and DNA filters were hybridized with nick-translated probe DNAs (RIGBY *et al.* 1977) according to the procedure of ENDOW (1982). Hybridized filters that had been washed and air dried were autoradiographed in X-ray cassettes using Kodak XRP-1 film covered with DuPont Cronex Lightning Plus intensifying screens.

Autoradiographs of dot blot hybrids were aligned over their respective filters, and the regions delimiting areas of hybridization were marked by poking through the film, and subsequently through the filter, with a needle probe. The areas of the filter containing radioactively labeled dots were cut out with a cork borer and were placed individually into scintillation vials.

RESULTS

Before performing the quantitative experiments on *Sgs-4* RNA production, RNA samples from each type of male and female described above were subjected to gel electrophoresis, blotted to nitrocellulose filters, and hybridized to a mixture of ^{32}P-labeled pkdm4B1 and λcDm2008 homologous to *Sgs-4* and *Sgs-3* RNA, respectively. Autoradiographs were made from the filters, and these were used to demonstrate that each type of larval salivary gland studied produced both the 950 base *Sgs-4* RNA and the 1100 base *Sgs-3* RNA, as was the case for all larvae, noting that in H/Y males just detectable amounts of *Sgs-4* RNA are found.

Dosage compensation of *Sgs-4*: In order to determine the sensitivity of our RNA assay, we performed a series of experiments designed to confirm the dosage compensation of the *Sgs-4* gene product, *i.e.*, its equivalence between males and females, as well as the customary dosage dependence within a sex. An Oregon-R wild-type strain (OR) was used for the normal male and female comparisons. A Hikone wild-type strain (H) that accumulates only trace amounts of *Sgs-4* protein (KORGE 1981) and transcript (MUSKAVITCH and HOG-

TABLE 1

Dosage effects on steady-state levels of *Sgs-4* transcripts

Constitution	Relative no. of *Sgs-4* genes[a]	Relative no. of *Sgs-3* genes	*Sgs-4*/*Sgs-3* transcripts[b]	n^c	95% confidence limits for the difference between the means
OR/OR (♀)	2	2	1.56 ± 0.04 (1.14, 1.86)	2	(−0.001, 0.041)
OR/Y (♂)	1	2	1.58 ± 0.10 (1.33, 1.83)	3	
OR/H (♀)	1	2	2.43 ± 0.06 (1.89, 2.98)	2	(−2.28, −2.24)
H/Y (♂)	0	2	0.17 ± 0.06 (0.00, 0.71)	2	
OR/H (♀)	1	2	1.74 ± 0.22 (1.19, 2.29)	3	(2.05, 2.17)
OR/Y (♂)	1	2	3.85 ± 0.06 (3.70, 4.00)	3	
OR/*gt* w^a (♀)	2	2	1.60 ± 0.15 (1.23, 1.97)	3	(0.93, 1.11)
OR/Yw^+ (♂)	2	2	2.62 ± 0.24 (2.02, 3.22)	3	
H/*gt* w^a (♀)	1	2	2.20 ± 0.18 (1.91, 2.49)	4	(2.13, 2.35)
H/Yw^+ (♂)	1	2	4.44 ± 0.30 (3.96, 4.92)	4	

[a] The numbers represent fully active genes.
[b] Mean ratios of the counts hybridized to *Sgs-4* RNA and *Sgs-3* RNA and their standard error. The values in parentheses under each mean represent the 95% confidence interval.
[c] Number of independent RNA extractions.

NESS 1980) was used to generate single-dose females. Finally, the w^+Y chromosome (see LINDSLEY and GRELL 1968) was used to obtain males with two doses of *Sgs-4*.

Table 1 gives the results of the analysis of steady-state RNAs from the different types of males and females. There is no significant difference between the ratios of *Sgs-4* RNA/*Sgs-3* RNA in Oregon-R males and females. The 95% confidence interval of the difference of the means (−0.001, 0.041) suggests that a random sample of such male and female ratios will give a male mean only slightly greater or lower than the female mean.

The 95% confidence interval of the difference of the male and female means for OR/Y males and their OR/H sisters (2.05, 2.17), suggests that the males produce 215–225% as much *Sgs-4* RNA (relative to *Sgs-3* RNA) as do the females. This is somewhat greater than expected; however, this result indicates that females show a dosage response to active *Sgs-4* genes, and that the two different *Sgs-4* alleles probably operate autonomously. H/Y males exhibit 6.0–7.5% of the amount of *Sgs-4* RNA present in their OR/H sisters. OR/w^+Y males exhibit 158–170% of the *Sgs-4* RNA present in OR/*gt* w^a females (the

TABLE 2

Effect of *mle* alleles on steady-state levels of *Sgs-4* transcripts

Constitution	Sex	*Sgs-4/Sgs-3* transcripts	*n*	95% confidence limits for the difference between the means
mle^{ts}/mle^{ts} (18°)	♀	1.81 ± 0.17 (1.63, 1.99)	6	(−0.35, −0.29)
	♂	1.49 ± 0.10 (1.37, 1.61)	5	
mle^{ts}/mle^{185} (18°)	♀	3.32 ± 0.09 (3,21, 3.43)	5	(−0.11, 0.01)
	♂	3.27 ± 0.27 (2.93, 3.61)	5	
mle^{ts}/mle^{185} (29°)	♀	2.80 ± 0.43 (2.12, 3.48)	4	(−1.00, −0.68)
	♂	1.96 ± 0.18 (1.74, 2.18)	5	

See Table 1 for an explanation of column headings.

gt w^a *X* chromosome bears a wild-type allele of *Sgs-4*), while H/w^+Y males have 195–207% as much RNA as do their H/*gt* w^a sisters. These results show a reasonably normal level of activity for the *Sgs-4* gene in the *Y*-linked duplication and show a dose-response in males. It should be noted that the magnitude of the ratios seen in Table 1 varies among the different sibling pairs due to the differences in the specific activities of the probes used in separate experiments.

Effect of *mle* on the expression of *Sgs-4*: The first test of the effect of a male-specific lethal mutant allele on the level of *Sgs-4* transcript was performed on male larvae homozygous for mle^{ts}. Because of the necessity of obtaining mutant individuals with functional salivary glands, larvae were reared at 18°. This "permissive" temperature allows the survival of some, but not all, of the expected mutant males (BELOTE and LUCCHESI 1980b). In Table 2 it can be seen that mle^{ts}/mle^{ts} males possess 80–84% of the *Sgs-4* RNA found in homozygous mutant females. This difference, although significant, is smaller than that reported by BELOTE and LUCCHESI (1980a) and is probably due to the problem of having to select mutant larvae sufficiently developed to exhibit salivary gland secretion polypeptides synthesis. Furthermore, a greater proportion of larvae destined to be escapers may have been selected by the morphological criteria used in our experiments than were selected by BELOTE and LUCCHESI on the basis of polytene chromosome morphology. In an attempt to alleviate this problem, we used the heteroallelic combination mle^{ts}/mle^{185}.

The mutant allele mle^{185} was recovered in a screen where EMS-treated chromosomes were tested for male-specific lethality over an *mle*-bearing chromosome. Males of the genotype mle^{ts}/mle^{185} raised at 18° are unaffected by the mutations: they eclose in numbers equivalent to their heteroallelic sisters and develop at a rate characteristic of wild type. At 29° these males are all severely affected, although a number of them which have initiated salivary gland secretion synthesis can be selected. (The phenotype of homozygous mle^{185} individ-

TABLE 3

Quantitation of *Sgs-4* DNA sequences

Constitution	Sex	Sgs-4/Sgs-3[a] sequences	n[b]
OR	♀	1.13 ± 0.06	4
	♂	0.59	1
mle^{ts}/mle^{ts} (18°)	♀	1.05 ± 0.05	2
	♂	0.52 ± 0.04	2
mle^{ts}/mle^{185} (29°)	♀	1.12 ± 0.05	4
	♂	0.56	1

[a] Mean ratio of counts hybridized to *Sgs-4* and *Sgs-3* DNA and their standard errors.
[b] Number of independent DNA extractions.

uals has not been studied because attempts to remove co-induced lethal mutations have been unsuccessful to date.)

At 18° there is no significant difference in *Sgs-4* RNA production between mle^{185}/mle^{ts} males and females (Table 2). However, when data are collected from the same type of males and females raised at the restrictive temperature of 29°, the male level of *Sgs-4* RNA is 65–75% of the female level.

In order to demonstrate that the effect of *mle* on the dosage compensation of *Sgs-4* is not due to preferential underreplication of this gene or of the *X* chromosome, the relative level of *Sgs-4* to *Sgs-3* salivary gland DNA was compared between males and females by analyzing dot blot filters in a manner similar to that used for the RNA analysis. The data in Table 3 suggest that both mle^{ts}/mle^{ts} and mle^{185}/mle^{ts} males have the same amount of *Sgs-4* DNA compared to their sisters as do Oregon-R males.

DISCUSSION

BIRCHLER, OWENBY and JACOBSON (1982) measured the steady-state level of serine-4 transfer RNA in a dosage series for the *X*-linked coding site of this RNA and found that each gene dose has a greater expression in males than in females. With respect to RNA polymerase II transcripts, S. BECKENDORF (personal communications) has reported the equivalence of *Sgs-4* transcripts in males and females. GANGULY, GANGULY and MANNING (1985) have measured glucose-6-phosphate dehydrogenase (G6PD) messenger RNA levels in male and female adults and have found them to be equivalent. All of these observations support the conclusion of MUKHERJEE and BEERMANN (1965) that dosage compensation operates at the level of transcription.

Male-specific lethal mutations have been shown to alter total *X*-chromosome RNA synthesis and the relative activity of *X*-linked enzymes. Because of these effects, these mutations are thought to impair the activity of genes responsible or involved in dosage compensation. The major focus of our research was to determine if male-specific lethal mutations exert their action by altering the levels of specific transcripts. The *X*-linked *Sgs-4* gene was selected for study because it produces an abundant larval RNA, albeit during a short develop-

mental period, and because of the availability of a closely related autosomal gene, *Sgs-3*, which could serve as an internal control.

Sgs-4 RNA levels show dosage compensation as well as dosage response in females or males. In duplication-bearing males with two active doses of the gene (OR/w^+Y) the RNA level is not quite twice that in normal females and males. This cannot be ascribed to the gene in the duplication since in another genotype (H/w^+Y) it appears to be fully active and compensated. A possible explanation is that there may be a physiological upper limit on the amount of *Sgs-4* RNA that can be manufactured, processed or protected from degradation at any one time.

Sgs-4 RNA levels are moderately reduced in mle^{ts}/mle^{ts} and substantially reduced in mle^{ts}/mle^{185} males raised at 29°. Since the mle^{ts} and mle^{185} mutant alleles were induced in different genetic backgrounds, and since the heteroallelic combination studied was obtained by hybridizing the two mutant strains, we conclude that the observed effects are due to mutations in the *mle* gene. These results indicate that the expression of a wild-type *mle* product is necessary for complete dosage compensation of the *Sgs-4* transcript, and they lend strong support to the theory that *mle* exerts its lethal effect on males by acting to decrease the transcriptional activity of the *X*-chromosome.

The observation that mle^{ts}/mle^{185} males are fully viable when raised at 18° and that their *Sgs-4* genes are transcribed at a fully compensated level would suggest that this combination of mutations is capable of producing functional products. Ascertaining what is the basis for the temperature-sensitive effect seen with this heteroallelic combination might shed some light on the function of this male-specific lethal locus.

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